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MINI-REVIEW

The Possible Role of Redox-Associated Protons in Growth of Plant Cells

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Abstract

The protons excreted by plant cells may arise by two different mechanisms: (1) by the action of the plasma membrane H^+-ATPase and (2) by plasma membrane redox reactions. The exact proportion from each source is not known, but the plasma membrane H^+-ATPase is, by far, the major contributor to proton efflux. There is still some question of whether the redox-associated protons produced by NADH oxidation on the inner side of the plasma membrane traverse the membrane in a 1 : 1 relationship with electrons generated in the redox reactions. Membrane depolarization observed in the presence of ferricyanide reduction by plasma membranes of whole cells or tissues or the lag period between ferricyanide reduction and medium acidification argue that only scalar protons may be involved. The other major argument against tight coupling between protons and electrons involves the concept of strong charge compensation. When ferricyanide is reduced to ferrocyanide on the outside of cells or tissues, an extra negative charge arises, which is compensated for by the release of H^+ or K^+, so that the total ratio of increased H^+ plus K^+ equals the electrons transferred by transmembrane electron transport. These are strong arguments against a tight coupling between electrons and protons excreted by the plasma membrane. On the other hand, there is no question that inhibitor studies provide evidence for two mechanisms of proton generation by plasma membranes. When the H^+-ATPase activity is totally inhibited, the addition of ferricyanide induces a burst of extra proton excretion, or *vice versa*, when plasma membrane redox reactions are inhibited, the H^+-ATPase can function normally. Since plasma membrane redox reactions and associated H^+ excretion are related to growth, it is possible that in plants the ATPase-generated protons have a different function from redox-associated protons. The H^+-ATPase-generated protons have been considered for many years to be necessary for cell wall expansion, allowing elongation to take place. A special function of the redox-generated protons may be in initiating proliferative cell growth, based on the presence of a hormone-stimulated NADH oxidase in membranes of soybean hypocotyls

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and stimulation of root growth by low concentrations of oxidants. Here we propose that this NADH oxidase and the redox protons released by its action control growth. The mechanism for this may be the evolution of protons into a special membrane domain, from which a signal to initiate cell proliferation may originate, independent of the action of the H^+-ATPase-generated protons. It is also possible that both expansion and proliferative growth are controlled by redox-generated protons.

Key Words: Plasma membrane; proton excretion; H^+-ATPase-modulated protons; plasma membrane redox-associated protons; proton involvement in plant growth.

Introduction

Proton excretion by plant cells was well established before the discovery of the plasma membrane Mg^{2+}-ATPase (Serrano, 1985, 1988; Sze, 1985), but assumptions still have to be made regarding the precise mechanism of H^+ excretion. According to previous reviews on H^+ excretion by plasma membranes (Marré and Ballarin-Denti, 1985; Lüttge and Clarkson, 1985; Møller and Lin, 1986; Dahse *et al.*, 1989; Crane, 1989; Crane and Barr, 1989), the most important assumption is the extent of coupling between electron and proton transport across the plasmalemma. A non-electrogenic ratio of 1 : 1 is expected if an electron is accompanied by a proton across the membrane. The evidence on this point is not unequivocal; studies which provide evidence of no coupling between electron and proton transport include those of Rubinstein and Stern (1986), Del Valle-Tascon *et al.* (1987), Marré *et al.* (1988a, b), Trockner and Marré (1988), Guern and Ullrich-Eberius (1988), Ullrich and Guern (1990), and Bernstein *et al.* (1989). On the other hand, tight coupling between electrons and protons transported across the plasmalemma was found by Ivankina *et al.* (1984) with Elodea cells, by Neufeld and Bown (1987), Bown and Crawford (1988) and by Bown *et al.* (1988) with green asparagus suspension culture cells in the light and by Craig and Crane (1981) and by Barr (1988) with cultured carrot cells. Indirect support in favor of the electrogenicity of the plasmalemma proton pump is provided by Löppert (1983) and by Böttger *et al.* (1985) showing that ATPase alone could not explain changes in proton release when different oxygen tensions were used as the experimental variable. Elzenga and Prins (1989) in studies of light-induced polar pH changes in Elodea leaves came to the conclusion that the ferricyanide-induced H^+ extrusion and the H^+ transport during the polar reaction were mediated by different mechanisms. Various models of proton and electron coupling are well described and discussed by Guern and Ullrich-Eberius (1988). In this communication, a sampling of proton excretion rates in various organisms will be presented with emphasis on inhibitor studies, which clearly establish that the protons excreted by plant

cells arise from two different sources: (1) the action of the plasma membrane H^+-ATPase and (2) plasma membrane redox reactions.

Methods for Studying H^+ Excretion

External Measurement of pH with Whole Cells

It is easy to monitor the external pH of cells grown in tissue culture or suspended tissue segments by a combination pH electrode and a pH meter. This has been done with cultured carrot cells (Craig and Crane, 1981, 1985; Crane *et al.*, 1984; Barr, 1987, 1988), cultured mesophyll cells of *Asparagus* (Bown, 1982; Neufeld and Bown, 1987, Bown *et al.*, 1988; Bown and Crawford, 1988), with sugarcane cells (Thom and Maretzki, 1985; Komor *et al.*, 1987), *Catharanthus roseus* cells (Marigo and Belkoura, 1985; Belkoura and Marigo, 1986; Belkoura *et al.*, 1986), sycamore cells (Blein, 1981, 1982; Blein *et al.*, 1986), corn coleoptiles (Böttger *et al.*, 1985), roots of *Zea mays* (Federico and Giartosio, 1983; Böttger and Lüthen, 1986; Rubinstein and Stern, 1986; Böttger and Hilgendorf, 1988; Lüthen and Böttger, 1988), roots of iron-deficient bean plants (Sijmons and Bienfait, 1986; De Vos *et al.*, 1986), dwarf bean plants (Van Beusichem *et al.*, 1988), roots of iron-deficient cucumber (Alcántara and de la Guardia, 1988), sunflower hypocotyls (Böttger *et al.*, 1984), intact sunflower plants (Römheld *et al.*, 1984), *Elodea* (Novak and Ivankina, 1978, 1983; Ivankina *et al.*, 1984; Ivankina and Novak, 1988; Novak *et al.*, 1988; Marré *et al.*, 1988; Trockner and Marré, 1988), *Lemna gibba* (Lass *et al.*, 1986; Guern and Ullrich-Eberius, 1988; Ullrich and Guern, 1990), and *Lamprothamnium papulosum* (Thiel and Kirst, 1988). The theory of pH measurement is provided by Henderson and Graf (1988).

Measurement of Proton Movement in Isolated Plasma Membrane Vesicles. The techniques for proton movement in isolated membrane vesicles were first developed in animal systems (Lee *et al.*, 1982) using fluorescent probes, 9-aminoacridine, acridine orange, quinacrine, or neutral red. These techniques were then applied to plant membrane vesicles to study proton movements controlled by ATPases (Sze, 1985). Examples of recent studies using quinacrine fluorescence include studies by Galtier *et al.* (1988) and DuPont (1989), and with acridine orange fluorescence by DuPont (1989). Shortcomings of this technique are described by Pope and Leight (1988) and by Grzesiek and Dencher (1988). There is only one study of redox-associated H^+ movements in isolated plasma membranes by fluorescent dye techniques (Giannini and Briskin, 1988), which yielded negative results with plasma membranes from red beet storage tissue. The authors attempted to incorporate ferricyanide into vesicles by a freeze-thaw method, but could not detect any redox-associated H^+

movement inward, unless detergent was added to the preparation. Reversal of the process to pump protons out by Böttger (1989) was successful. By electroporation, he incorporated NADH into plasma membrane vesicles prepared from soybean hypocotyls and added ferricyanide on the outside. This resulted in an electron and H^+ transfer from the cytoplasmic to the apoplastic side of these vesicles. The rate of electron transfer could be stimulated by a low concentration of uncouplers or by Ca^{2+} ions.

Hassidim *et al.* (1987) developed a system with plasmalemma-enriched membrane vesicles from cotton and radish seedling roots, whereby transmembrane electron transport from ascorbate (inside) or ferricyanide (outside) could be observed along with accumulation of protons inside these vesicles.

As H^+ are excreted from cells by a coupled process, the internal pH of cells should become more alkaline. Böttger's (1989) study with vesicles indicates an internal pH increase, since the reaction is favored by acidic pH inside the vesicle. Guern *et al.* (1988) show increase of cytosolic pH during ferricyanide reduction by intact cells.

Proton Excretion Associated with Plasmamembrane Redox from Various Plants

Proton excretion associated with plasma membrane redox reactions has been studied mainly in plant roots (Federico and Giartosio, 1983; Sijmons *et al.*, 1984; Böttger and Lüthen, 1986; Rubinstein and Stern, 1986; Lüthen and Böttger, 1988; Böttger and Hilgendorf, 1988), and in aquatic plants, *Elodea* and *Lemna* (Ivankina and Novak, 1980, 1988; Novak and Ivankina, 1983; Ivankina *et al.*, 1984; Lass *et al.*, 1986; Marré *et al.*, 1988a and b; Guern and Ullrich-Eberius, 1988; Ullrich and Guern, 1980), as well as in cell cultures. These include carrot (Craig and Crane, 1982, 1985; Crane *et al.*, 1985; Barr, 1987 and 1988; Chalmers *et al.*, 1984), *Catharanthus roseus* (Marigo and Belkoura, 1985; Guern *et al.*, 1988) and *Asparagus sprengeri* (Neufeld and Bown, 1987; Bown and Crawford, 1988; Bown *et al.*, 1988; Pugin *et al.*, 1988).

A sample of proton excretion rates associated with transplasmalemma electron transport by various plants or cells is reported in Table I. These rates are above or in addition to the basal H^+ excretion rates often attributed to the plasma membrane H^+-ATPase. They were obtained by adding ferricyanide to the reaction medium as the nonpermeable electron acceptor on the outside of plant roots or cells. The basal rate may also include contribution from the NADH oxidase in the membrane, but methods for analysis of this contribution are still in development (Böttger and Lüthen, 1986; Ivankina and Novak, 1988; Xia and Saglio, 1990).

Table I. Ratios of Protons Excreted to Ferricyanide Reduced by Plant Cells or Tissues

Plant tissue	Units for measurement	H^+ excretion with HCF III (Δ)	HCF III reduced	Ratio H^+/e^-	Reference
Asparagus sprengeri Regel	nmol $(10^6$ cells$)^{-1}$ min^{-1}				
Mesophyll cells		1.54	1.55	1.0	Neufeld and Bown, 1987
Mesophyll cells in the light		11.8	10.4	1.14	
		13.1	12.3	1.07	Bown *et al.*, 1988
		33.5	31.8	1.05	
Carrot cells	nmol mg^{-1} DW min^{-1}	6.01 ± 0.99	4.83 ± 0.20	1.24	Crane *et al.*, 1983
Carrot cells	nmol mg^{-1} DW min^{-1}	3.8	3.5	1.09	Craig and Crane, 1985
Catharanthus roseus cells	nmol or ΔpH h^{-1} 10^6 protoplasts^{-1}	0.36	396	≈1	Marigo and Belkoura, 1985
Cuscuta apical segments	μmol min^{-1} g^{-1} DW	0.59 ± 0.1	4.55 ± 0.16	0.13	Revis and Misra, 1988
Elodea canadensis leaves	—	—	—	1.05 ± 0.04	Novak and Miklashevich, 1984
Elodea densa	μmol g^{-1} FW 90 min^{-1}	28.3	13.4	2.11	Marré *et al.*, 1988b
Lamprothamnium papulosum	—	3.1 ± 0.6	3.7 ± 0.7	0.8	Thiel and Kirst, 1988
Lemna gibba G1					
+Fe	μmol g^{-1} FW H^{-1}	1.26 ± 0.17	2.17 ± 0.51	0.58 ± 0.12	Lass *et al.*, 1986
−Fe	μmol g^{-1} FW H^{-1}	1.64 ± 0.34	2.57 ± 0.47	0.64 ± 0.13	
Lemna gibba fronds in					
$CaSO_4$	μmol or μequiv. h^{-1} g^{-1} FW	8.6 ± 0.5	10.6 ± 0.8	0.81	Guern and Ullrich-Eberius, 1988
In 1X medium	μmol or μequiv. h^{-1} g^{-1} FW	5.7 ± 0.7	10.5 ± 0.9	0.54	
Maize roots					
Aerobiosis	μmol h^{-1} g^{-1} FW	0.49	1.20	0.41	Federico and Giartosio, 1983
Anaaerobiosis	μmol h^{-1} g^{-1} FW	0.03	1.0	0.03	
Maize root segments	μmol g^{-1} FW h^{-1}	1.0	4.5	0.22	Rubinstein and Stern, 1986
Maize roots	—	—	—	1.0	Böttger and Hilgendorf, 1988
Phaseolus vulgaris					
Iron-sufficient	μmol h^{-1} g^{-1} FW	—	3.7	1.0	Van Beusichem *et al.*, 1988
Iron-deficient	μmol h^{-1} g^{-1} FW	—	—	0.5	
Sugarcane protoplasts	μmol h^{-1} $(10^6$ protoplasts$)^{-1}$	0.39	1.0	0.39	Komor *et al.*, 1987
Sycamore cells	nmol or μequiv. min^{-1} mg^{-1} DW	3.74	3.68	1.02	Blein *et al.*, 1986
Sycamore cells	nmol min^{-1} g^{-1} FW	370	464	1.25	Pugin *et al.*, 1988
Vicia faba					
Roots	μmol h^{-1} FW^{-1}	5.0	10.1	0.50	Sijmons *et al.*, 1984
Iron-deficient	μmol h^{-1} FW^{-1}	3.0	5.3	0.57	
Iron-deficient	μmol h^{-1} FW^{-1}	5.7	9.5	0.60	

Sources of H^+ Excretion

H^+-ATPase

There is no question that the majority of H^+ excreted by aquatic plants, plant roots, or cultured cells is mediated by the plasma membrane H^+-ATPase (Serrano, 1985, 1988; Sze, 1985). The enzyme, a 100-kDa protein or possibly an oligomer of the basic subunit, which consists of eight transmembrane loops, excretes protons through a hydrophobic channel through the plasma membrane to the outside of the cell. The excreted protons may participate in cell wall loosening according to the acid growth hypothesis, introduced by Hager *et al.* (1971) and Cleland (1976).

The extra protons released in the presence of ferricyanide, when trans-plasma membrane electron transport takes place, have been interpreted in several ways. Originally (Craig and Crane, 1981; Federico and Giartosio, 1983), it was proposed that every electron crossing the plasma membrane was accompanied by a proton, i.e., there was tight coupling between electrons and protons, as in the mitochondria or chloroplast electron transport chains. Then Rubinstein and Stern (1986) discovered a lag of 5 min between ferricyanide reduction and proton excretion by maize roots. They proposed that only electrons cross the plasma membrane, while the extra protons generated by NADH oxidation stimulate the H^+-ATPase and its channel is used for H^+ excretion from the cell. These two extreme cases, tight coupling between protons and electrons versus no coupling, and models in between have been thoroughly described and discussed by Guern and Ullrich-Eberius (1988). Working with *Lemna gibba*, these authors find H^+/e^- ratios which are lower than 1 for ferricyanide reduced versus H^+ excreted. In the case of tight coupling, the ratio between H^+ excreted and ferricyanide reduced should be 1. Examining the H^+/e^- ratio reported in Table I, it can be seen that they are close to 1 in cultured carrot and Asparagus mesophyll cells. On the other hand, they vary more with *Elodea* or *Lemna gibba* and with corn roots. As pointed out by Guern and Ullrich-Eberius (1988) and Ullrich and Guern (1990), the variability between H^+ excreted and ferricyanide reduced by *Lemna* may depend a great deal on the pretreatment or the nutritional status of these organisms. Furthermore, unity between H^+ excreted and ferricyanide reduced is reached when K^+ efflux is taken into account, i.e., H^+ and K^+ efflux together equal ferricyanide reduction rates. Rubinstein and Stern (1986) found that H^+/e^- ratios depended on the age of the roots used, the composition of the reaction medium and, especially, the point in time when the H^+ excretion rates were determined. Generally, H^+ excretion rates were high in the initial 5–10 min but reached a steady state during the first 10–30 min. Therefore, the H^+/e^- ratios can vary a great deal, unless caution

is taken in comparing electron transport and H^+ excretion rates under identical conditions and at a common point in time. When both measurements are made simultaneously on the same sample in the same reaction mixture, a 1 : 1 ratio between H^+ excreted and ferricyanide reduced can be obtained with cultured carrot cells (Craig and Crane, 1985).

Inhibitors of H^+ Excretion

It is possible to selectively inhibit or stimulate one or the other mechanism of H^+ excretion by plant cells. The basal rate, often attributed to the action of the plasmalemma H^+-ATPase, can be partially or completely inhibited by ATPase inhibitors, sodium vanadate, DCCD, diethylstilbestrol, or erythrosine B (Sze, 1985; Marré *et al.*, 1988a, b; Bown and Crawford, 1988; Bown *et al.*, 1988; Rubinstein and Stern, 1986; Hassidim *et al.*, 1987; Barr *et al.*, 1987). These inhibitors have less effect on the H^+ excretion associated with transmembrane ferricyanide reduction over a few minutes, but they can be inhibitory to ferricyanide reduction over longer periods (Rubinstein and Stern, 1986).

Mitochondrial electron transport inhibitors (sodium azide, KCN, HOQNO, antimycin A) usually inhibit the basal rate of H^+ excretion, presumably by reducing the supply of ATP available for ATPase action, although sometimes preincubation with the inhibitors for up to 30 min is necessary. Mitochondrial electron transport inhibitors show only slight effects on transplasmalemma electron transport-associated H^+ excretion (Barr, 1987). However, these redox-associated protons can be inhibited to varying degrees by plasma membrane electron transport inhibitors (anthracycline drugs, actinomycin d) and by calmodulin antagonists (Barr *et al.*, 1985b). Uncouplers can have various effects on both types of H^+ excretion. This is also true for ions, depending on their concentration and length of contact with cells or tissues. Examples of these various classes of inhibitors and their action will be provided in the following sections.

The Effect of ATPase Inhibitors on H^+ Excretion

The modulation of H^+ excretion by the plasma membrane H^+-ATPase as the only mechanism of H^+ excretion by plant cells has been questioned (Lüttge and Clarkson, 1985; Møller and Lin, 1986; Rasi-Caldogno *et al.*, 1986). Somehow, the presence of an impermeable electron acceptor on the outside of the plasma membrane induces an additional increment of H^+ excretion observable by acidification of the outside medium. The origin of these extra protons is still controversial. The simplest explanation favored by the early investigators of plasma membrane electron transport (Craig and

Crane, 1982; Federico and Giartosio, 1983) was to assume that the extra protons are tightly coupled to the oxidation of the natural electron donor, NAD(P)H, and that they are transported to the outside of the plasma membrane through unique proton channels along with the electrons using the plasma membrane electron transport chain. However, after Rubinstein and Stern (1986) noticed a lag period of 5 min before acidification of the bathing solution of maize roots after ferricyanide reduction, they proposed that only electrons traverse the plasma membrane unaccompanied by protons. In that case, as also envisioned by Morré *et al.* (1986), Lass *et al.* (1986), and Marré and associates (Marré *et al.*, 1988b; Trockner and Marré, 1988), the protons generated by NAD(P)H oxidation would remain on the cytoplasmic side to acidify the cytoplasm and thereby stimulate the H^+-ATPase to action to excrete extra protons to the outside of the cell through its regular proton channel. Under salt stress, ^{31}P NMR spectroscopy can show cytoplasmic acidification and vacuolar alkalinization in *Nitellopsis obtusa* cells (Katsuhara *et al.*, 1989), but other investigators using ^{31}P NMR spectroscopy can find no acidification of the cytoplasm in undisturbed tissue in the presence of ferricyanide in contrast to using expressed cell sap pH measurements, where the acidification seen could have arisen from mixing the contents of broken cell compartments (Marré *et al.*, 1988b). Guern *et al.* (1988) have described internal acidification during ferricyanide reduction using ^{31}P NMR. Oscillations in the pH of 20–30 min duration have been shown with the epidermal cells of maize coleoptiles (Felle, 1988). Senn and Goldsmith (1988) did not find cytoplasmic acidification until 40 min after application of auxin or fusicoccin with oat coleoptiles. The other supporting claim in favor of the H^+-ATPase as the only proton source comes from Rubinstein and Stern's (1986) observations that ATPase inhibitors inhibit proton excretion by maize roots in presence of ferricyanide. Redox proton excretion is also inhibited by ATPase inhibitors in carrot cells (Table II), while ferricyanide reduction is inhibited to a lesser degree in these cells. In complete contrast to maize roots and carrot cells are studies with cultured asparagus cells (Neufeld and Bown, 1987; Bown and Crawford, 1988). Here the authors show that when ATPase action is completely blocked by ATPase inhibitors, the addition of ferricyanide induces a fresh burst of H^+ excretion.

In summary, the inhibition of H^+ excretion associated with redox reactions of the plasma membrane in plant cells by ATPase inhibitors does not conclusively prove that the protons originated from the same basic source, since DCCD, for example, does not inhibit the H^+-ATPase of the plasma membranes exclusively but also those of chloroplasts (McCarty, 1977) and mitochondria (Beechey *et al.*, 1966). Therefore, if perchance DCCD also inhibits the redox-associated H^+ release in plasma membranes, it should not surprise anyone. At present, it cannot be excluded that the redox-generated

Table II. The Effect of ATPase Modulators on H^+ Excretion Associated with Transplasmalemma Electron Transport

Plant material	Modulator	Concentration (μM)	Inhibition or stimulation of H^+ with HCF III (Δ) (%)	Inhibition or stimulation of HCF III reduction (%)	Reference
Asparagus sprengeri cells	DES	100			
Light			−25	—	Bown and
Dark			−12	—	Crawford, 1988
Carrot cells	DCCD	100	−43	—	Barr, 1987
Carrot cells	DES	50	−98	—	
Carrot cells	Sodium vanadate	100	−100	—	
Carrot cells	DCCD	300	−55	−50	Barr *et al.*, 1987
Carrot cells	DES	30	−69	−70	
Catharanthus roseus cells	Sodium vanadate	200	−51	−3	Belkoura and Marigo, 1986
Corn root apical segments	DCCD	50	−100	−50	Rubinstein and Stern, 1986
Corn root apical segments	DES	50	−80	−100	
Corn root apical segments	Sodium vanadate	100	−75	−40	
Elodea densa	Erythrosine B	100	−79	−13	Marré *et al.*,
	Sodium vanadate	100	−60	+1	1988b
Maize roots					
Aerobiosis	Fusicoccin	10	+6.35x	+21	Federico and
Anaaerobiosis	Fusicoccin	10	+10x	+17	Giartosio, 1983

protons use the ATPase channels to migrate to the outside of the cell, if the two enzymes, the H^+-ATPase and the inner NADH oxidase, are located adjacent to each other in the membrane. Likewise, it cannot be concluded in this stage of development that one single transmembrane enzyme does everything, including transmembrane electron transport and total H^+ excretion.

The Effect of Mitochondria Electron Transport Inhibitors on H^+ Excretion

The most dramatic effect on the action of the H^+-ATPase of plant plasma membranes would be total deprivation of ATP. None of the mitochondria electron transport inhibitors reported in Table III would be expected to do that within the time limits of the assay, 10 min. However, high concentrations of oligomycin (50 μg/ml), which specifically inhibit the F_1 mitochondrial ATPase (DeRobertis and DeRobertis, Jr., 1987) gave 100% inhibition of the basal rate of the plasma membrane H^+-ATPase, whereas it stimulated the redox-associated H^+ excretion rate in carrot cells. HOQNO, a lipophilic compound which interferes with the non-heme iron function of complex III in mitochondria (Douce and Day, 1985), also inhibits the basal rate of H^+ excretion in carrot cells more than the redox-associated H^+ (100% inhibition versus 33% inhibition, Table III). Antimycin a, which inhibits at the same site as HOQNO in mitochondria, had less effect on both types of H^+ excretion in carrot cells. KCN, the best known inhibitor of cytochrome oxidase in mitochondria (Douce and Day, 1985), inhibited the basal rate of H^+ excretion in carrot cells from 66 to 90%, but it showed no inhibition in the presence of ferricyanide (Table III).

A direct comparison of H^+ excretion rates with ferricyanide reduction rates in the presence of mitochondrial electron transport inhibitors on the same cell culture at the same time is missing from published data with carrot cells, but a cursory examination of the data in Table III shows less correlation between the two activities than expected on a 1 : 1 basis. This, again, may indicate a more complex relationship between transmembrane ferricyanide reduction and proton excretion than direct tight coupling.

Hypoxic conditions also decrease cellular ATP so that all proton release is inhibited, but these conditions would also eliminate transmembrane NADPH oxidase activity. If 2-deoxyglucose is used to decrease ATP to the same levels as anoxia, proton efflux continues at 50% of the aerobic rate (Xia and Saglio, 1990). Since 2-deoxyglucose can be metabolized by the hexose monophosphate shunt to produce NADPH (M. G. Clark, personal communication), the NADPH oxidase may power the transport of protons without involvement of the ATPase.

Table III. The Effect of Mitochondria Electron Transport Inhibitors on H^+ Excretion and Ferricyanide Reduction Associated with Transplasmalemma Electron Transport

Plant material	Modulator	Concentration (μm)	Inhibition or stimulation of H^+ with HCF III (Δ) (%)	Inhibition or stimulation of HCF III reduction (%)	Reference
Carrot cells	KCN	1000	+27.5	+36	Misra and Crane, 1983
Carrot cells	Antimycin a	10	−26	−41.7	
Carrot cells	Sodium azide	700	−2.7	−40	
Carrot cells	KCN	200	0	—	Barr, 1987
Carrot cells	Antimycin a	3	−12	—	Barr and Crane, 1990
Carrot cells	NOQNO	60	−33	—	Barr, 1987
Carrot cells	Oligomycin	50 μg/ml	+66	—	
Carrot cells	Sodium azide	30	−64	—	
Carrot cells	KCN	1000	—	+36	Barr *et al.*, 1985a
Carrot cells	Antimycin a	1	—	−11.4	
Carrot cells	HOQNO	10	—	−5.3	
Carrot cells	Sodium azide	500	—	−18	

Table IV. The Effect of Protonophores on Transplasmalemma Electron Transport and Associated H^+ Excretion

Plant material	Modulator	Concentration (μM)	Inhibition of H^+ with HCF III (Δ) (%)	Inhibition of HCF III reduction (%)	Reference
Carrot cells	SF6847	1	−89	+40	Barr *et al.*, 1987
Carrot cells	CCCP	1	−67	−45	Barr, 1987
Carrot cells	FCCP	1.5	−98	−52	
Carrot cells	Desaspidin	3	−84	0	
Carrot cells	Gramicidin	3	−90	0	
Carrot cells	SF6847	1.5	−70	0	
Carrot cells	1799	0.3	−82	0	

The Effect of Protonophores on H^+ Excretion

Protonophores or uncouplers normally abolish the tight coupling between electron transport and ATP synthesis as in chloroplasts (Jagendorf, 1977) or in mitochondria (Douce and Day, 1985) by destroying the proton gradients across membranes (Heytler, 1979). As far as the ATPase of plasma membranes is concerned, its activity should be stimulated by ionophores (Sze, 1985), particularly with those such as nigericin which transport H^+ in exchange for K^+. The uncouplers, FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) and CCCP (carbonylcyanide *m*-chlorophenylhydrazone), which transport only H^+, might be inhibitory, as Table IV shows. On hindsight, the concentrations of uncouplers used in Table IV may be too high. The recommended concentrations to uncouple isolated mitochondria according to Heytler (1979) are as follows: SF 6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidinemalononitrile), 3×10^{-8} M; CCCP, 10^{-7} M; FCCP, 4×10^{-8} M; desaspidin, 2×10^{-7} M; 1799 [2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one], 3×10^{-6} M. The higher concentrations of uncouplers used by Barr (1987) or by Barr *et al.* (1987) were effective inhibitors of the basal rate of H^+ excretion by carrot cells, as well as on the redox-associated H^+ excretion in presence of ferricyanide, producing inhibitions from 70 to 98%. These uncouplers, with the exception of CCCP and FCCP, had little or no effect on transmembrane ferricyanide reduction (Table IV). The almost equal inhibition of both types of proton excretion by uncouplers favors the consideration of a common proton channel for both, ATPase-generated and redox-associated, H^+ excretion.

The Effect of Plasma Membrane Electron Transport Inhibitors on H^+ Excretion Associated with Ferricyanide Reduction

Transplasma membrane electron transport with ferricyanide as the impermeable electron acceptor is inhibited best by anthracycline drugs in

Table V. The Effect of Plasma Membrane Electron Transport Inhibitors on H^+ Excretion Associated with Ferricyanide Reduction

Plant material	Modulator[a]	Concentration (μM)	Inhibition or stimulation of H^+ with HCF III (Δ) (%)	Inhibition or stimulation of HCF III reduction (%)	Reference
Carrot cells	Adriamycin (3 min)	3	−43	−22	Barr (unpublished)
Carrot cells	Retinoic acid	0.001	−100	−40	
Carrot cells	Actinomycin d				
	(3 min)	10	—	−17	
	(60 min)	10	−100	−55	
Carrot cells	Nitrophenyl acetate				
	(3 min)	100	−10	−12	
Carrot cells	SITS (60 min)	100	−10	−18	
Carrot cells	EDAC (60 min)	100	−20	-47	
Carrot cells	cis-Plat (60 min)	50	−20	−25	

[a]SITS, 4-acetamido-4′-isothiocyanostilbene-2,2-disulfonate; EDAC, [*N*-ethyl-*N*′-(dimethylamino-propyl)-carbodiimide; cis-Plat, cis-diaminodichloroplatinum(II).

animal cells (Crane *et al.*, 1985). In plant cells, inhibition by anthracycline drugs is variable depending on the age of the cells. Adriamycin, a well-known antitumor drug, inhibits ferricyanide reduction by cultured carrot cells up to 40% (Table V). Adriamycin in concentrations over 10 μM begins to act as an electron acceptor and superoxide generator to stimulate the ferricyanide reduction rate. The use of lower concentrations in the range of 1–5 μM usually gives the maximum inhibition. Actinomycin D is not permeable to the plasma membrane, and is generally more reliable as an inhibitor of transmembrane ferricyanide reduction, but at 10 μM it requires a preincubation period from 10–60 min to obtain 50% inhibition. *p*-Nitrophenyl acetate, which inhibits transmembrane ferricyanide reduction well with isolated plasma membranes (Barr *et al.*, 1986), has no effect with whole cells. It is possible that esterases in the cell wall break it down, although it inhibits elongation growth by soybean hypocotyl segments (Morré *et al.*, 1988a, b) without difficulty. Incubation with 100 μM EDAC [*N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide] for 60 min can inhibit up to 50% of ferricyanide reduction by carrot cells (Table V), but SITS, an inhibitor of anion channels (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) is not so effective. An examination of Table V shows that EDAC and SITS also inhibit both types of proton excretion, the basal rate attributed to the plasma membrane H^+-ATPase and the redox-associated protons.

Plasma membrane electron transport inhibitors usually stimulate the action of the plasma membrane H^+-ATPase (no data given), but inhibit the excretion of redox-associated protons (Table V). The most effective plasma membrane redox and associated redox H^+ excretion inhibitor appears to be actinomycin D, followed by retinoic acid. The fact that plasma membrane redox inhibitors only inhibit the redox-associated proton release but stimulate the action of the H^+-ATPase is the best evidence for two sources of H^+ excretion by plant plasma membranes.

The Effect of Calmodulin Antagonists on H^+ Excretion

Calmodulin antagonists (Table VI) were originally shown to inhibit transmembrane ferricyanide reduction by carrot cells (Barr *et al.*, 1985b). A 10-min preincubation period was required to obtain from 33% inhibition with calmidazolium, to less with fluphenazine, pimozide, and trifluoperazine. In a later study (Barr *et al.*, 1990a) showed that H^+ excretion in presence of ferricyanide was inhibited far more severely than transmembrane ferricyanide reduction. Complete inhibition was given by 50 μM calmidazolium, chlorpromazine, and trifluoperazine, while H^+ excretion by the H^+-ATPase of plasma membranes was stimulated or inhibited to a lesser degree. These data imply that Ca^{2+} ions and calmodulin are modulating some aspect of H^+ excretion and electron transport in plasma membranes. Our use of calmodulin antagonists in the original studies of plasma membrane redox reactions had no basis other than the assumption that the signal generated by a plant hormone had to be transmitted to the inside of the cell by some means, and the inositol triphosphate cascade could be the preferred mechanism (Crane, 1989). Meanwhile, Collinge and Trewavas (1989) have isolated an EGTA-stable form of calmodulin from pea stem plasma membranes where it constitutes 0.5–1% of total plasma membrane protein.

The Effect of Herbicides on H^+ Excretion

The most complete study of herbicide effects on transmembrane ferricyanide reduction and associated H^+ excretion has been carried out with phenmedipham by Blein *et al.* (1986). They found 52% inhibition of ferricyanide reduction by sycamore cells and 66% inhibition of H^+ excretion in presence of ferricyanide (Table VII). After testing several analogs of phenmedipham, the authors found that two of them, AM 6 and AM 8, were even more effective than phenmedipham itself, resulting in 98–100% inhibitions of both transmembrane redox and associated H^+ excretion, indicating that plasma membrane electron transport reactions can be a new site for the action of herbicides.

Table VI. The Effect of Calmodulin Antagonists on Transplasmalemma Electron Transport and Associated H^+ Excretion

Plant material	Modulator[a]	Concentration (μM)	Inhibition or stimulation of H^+ with HCF III (Δ) (%)	Inhibition or stimulation of HCF III reduction (%)	Reference
Catharanthus roseus cells	Calmidazolium (20 min)	10 mmoles m^{-3}	—	−22	Belkoura *et al.*, 1986
Catharanthus roseus cells	(total ΔpH)	30 mmoles m^{-3}	−98	−98	
Carrot cells	Calmidazolium	50	−100	−33	Barr *et al.*, 1990a
Carrot cells	Chlorpromazine	50	−100	−62	
Carrot cells	Trifluoperazine	50	−100	−26	
Carrot cells	W-7	50	−22	−39	
Carrot cells	W-5	50	−61	+33	

[a]W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

Table VII. The Effect of Herbicides on Transplasmalemma Electron Transport and Associated H^+ Excretion

Plant material	Inhibitor	Concentration (μM)	Inhibition of H^+ with HCF III (Δ) (%)	Inhibition of HCF III reduction (%)	Reference
Sycamore cells	Phenmedipham	100	−66	−52	Blein *et al.*,
Sycamore cells	AM 6	100	−100	−98	1986
Sycamore cells	AM 8	100	−100	−100	
Maize root plasmalemma vesicles	Sethoxydim at pH 5	500	—	−52	Fisher *et al.*, 1988a
Poa pratensis plasmalemma vesicles	Sethoxydim at pH 5	500	—	−51	
Poa annua plasmalemma vesicles	Sethoxydim at pH 5	500	—	−80	
Sycamore cells	Deoxyphomenone	10	−30	−34	Pugin *et al.*,
		50	−45	−38	1988
		100	−57	−57	

The other herbicide, sethoxydim, a cyclohexanedione derivative which stops root and leaf growth, induces chlorosis and necrosis leading to death in susceptible species (Fischer *et al.*, 1988a, b), was found to inhibit transmembrane ferricyanide reduction of isolated plasma membrane vesicles of maize roots, *Poa pratensis*, a sensitive species, and *Poa annua*, a tolerant species, from 51 to 80% (Table VII), while H^+ excretion by the plasma membrane H^+-ATPase was totally unaffected by 500 μM sethoxydim. The fact that plasma membrane electron transport in the sensitive species, *Poa pratensis* was less affected by sethoxydim than in *Poa annua*, a tolerant species, is hard to explain, but H^+ excretion in presence of ferricyanide and sethoxydim was not measured to see if there was a difference between the sensitive and the tolerant species of *Poa*. Deoxyphomenone (Pugin *et al.*, 1988) showed a good correlation between HCF III reduction and HCF III-associated H^+ excretion (Table VII).

The Effect of Ions on H^+ Excretion

A systematic study of the effect of various mono-, di-, or trivalent ions on transmembrane ferricyanide reduction versus associated H^+ excretion is not available. Marigo and Belkoura (1985) provide the only useful data for *Catharanthus roseus* cells (Table VIII). Here, tested at 1 mM concentration, $CaCl_2$, $MgCl_2$, and $LaCl_2$ stimulated both ferricyanide reduction and H^+ excretion in presence of ferricyanide. The above were immediate effects, seen

Table VIII. The Effect of Ions on Transplasmalemma Electron Transport and Associated H^+ Excretion

Plant material	Ion	Concentration (μM)	Inhibition or stimulation of H^+ with HCF III (Δ) (%)	Inhibition or stimulation of HCF III reduction (%)	Reference
Catharanthus roseus cells	$CaCl_2$	1	+45	+59	Marigo and Belkoura, 1985
Catharanthus roseus cells	$MgCl_2$	1	+17	+18	
Catharanthus roseus cells	$MnCl_2$	1	—	−78	
Catharanthus roseus cells	$ZnCl_2$	1	—	0	
Catharanthus roseus cells	$LaCl_3$	1	+39	+50	
Catharanthus roseus cells	$AlCl_2$	1	—	+24	
Catharanthus roseus cells	KCl	100	—	−4	
Catharanthus roseus cells	NaCl	100	—	−3	

Table IX. The Effect of High NaCl Concentrations in the Culture Medium on Carrot Cell Redox Reactions and H^+ Excretion (Data from Barr and Crane, unpublished)

NaCl concentration (μM)	Inhibition of dry weight (%)	Inhibition of H^+ excretion		Inhibition of HCF III reduced (%)	Inhibition of NADH oxidized (%)	Inhibition of peroxidase activity (%)
		Basal rate (%)	HCF III (Δ) (%)			
100	56	30	91	73	100	85
200	66	31	100	87	100	94
300	64	43	100	89	100	95

on reaction rates within a few minutes during assays, but another aspect of redox reactions and associated H^+ excretion, tested by Barr and Crane on carrot cells in culture, was growth on high salt. In this experiment, carrot cells were grown for 7 days on the regular growth medium supplemented with 100, 200, and 300 mM NaCl. At the end of 7 days, cells were harvested, washed 3 times, and assayed for ferricyanide reduction, oxidation of external NADH, peroxidase activity, H^+ excretion, and dry weight. It was found that all of these parameters were inhibited (Table IX), with NADH oxidation producing 100% inhibition on 100 mM NaCl in the growth medium. Growth measured by dry weight was inhibited from 56–64%, while excretion of H^+ in presence of ferricyanide was inhibited from 91 to 100%. The least inhibited parameter tested was H^+ excretion by the plasma membrane H^+-ATPase (30–43% inhibition). These data support differences between H^+ excretion by the plasma membrane H^+-ATPase and redox-associated H^+ excretion.

Hormone Effects on H^+ Excretion

Two phases of growth in plants have to be distinguished, elongation growth (Cleland, 1976; Cleland and Rayle, 1978; Ray, 1987) and proliferation which includes cell division (DeRobertis and DeRobertis, Jr., 1987). The enzyme most likely involved in cell elongation is the hormone-sensitive NADH oxidase isolated from soybean hypocotyls by Brightman *et al.* (1988). This enzyme is inhibited by the plasma membrane redox inhibitors, particularly the anthracycline drugs, which also inhibit elongation growth bt soybean hypocotyls (Morré *et al.*, 1988). Proton excretion is presumed necessary for elongation growth according to the acid growth hypothesis (Cleland, 1976; Cleland and Rayle, 1978; Kutschera and Schopfer, 1985a), mediated by the plasma membrane H^+-ATPase (Sze, 1985; Serrano, 1985, 1988). There have been no systematic attempts to study H^+ excretion in presence of NADH, while the basal rate of H^+ excretion has been inhibited by ATPase inhibitors.

Sugarcane cells studied by Komor *et al.* (1987) did not oxidize extracellular NADH in absence of ferricyanide but sugarcane protoplasts did, showing that a few broken cells could have been involved in the NADH oxidation. Extra O_2 uptake and alkalinization of the medium were observed with sugarcane protoplasts. Kochian and Lucas (1985) also reported alkalinization of the medium with corn root segments or whole roots when NADH was added. These results bring up the question of whether H^+ are necessary for expansion growth.

The original view was (Cleland, 1980, 1987; Kutschera and Schopfer, 1985a) that H^+ were necessary for wall loosening during expansion growth and that acid or fusicoccin, which stimulated the H^+-ATPase of plasma membranes, could substitute for IAA-induced H^+ excretion. This is no longer the current view (Kutschera and Schopfer, 1985b). It has been suggested (Theologis *et al.*, 1985; Theologis, 1986; Hagen, 1987; Hagen *et al.*, 1984; Guilfoyle, 1986; Parthier, 1989) that one of the earliest auxin-induced response is the induction of several mRNAs. This is a fast response, which can be detected within 15–30 min after auxin application (van der Zaal *et al.*, 1987). Fusicoccin, which stimulates H^+ excretion by the plasma membrane H^+-ATPase (Marré *et al.*, 1973), was not effective in inducing the mRNAs associated with auxin application (Walker *et al.*, 1985). Although H^+ may have a role in cell wall loosening, it may be a secondary effect. Theologis *et al.* (1985) could inhibit the H^+-ATPase of pea epicotyl tissue with cerulenin, vanadate, or mannitol, which prevented cell elongation, but these compounds had no effect on the auxin-induced mRNA accumulation. Thus, it appears that a dichotomy in response to auxin may be recognized by the cell: (1) an immediate acidification of the apoplast, since basal and redox-mediated H^+ excretion are stimulated by auxin upon contact and (2) export processes of cell wall building blocks coded for by the rapidly induced mRNAs which appear 10–15 min after the application of auxin. Long-term effects of auxin are also known. In *Arabidopsis thaliana*, an auxin-induced gene encodes a DNA-binding protein, which is expressed several hours after the application of auxin (Alliotte *et al.*, 1989). The sequence of events proposed by Theologis (1986):

$$\text{mRNA induction} \longrightarrow H^+ \text{ secretion} \longrightarrow \text{cell elongation}$$

$$(10\text{–}15\,\text{min}) \qquad (15\text{–}25\,\text{min})$$

does not hold true in view of practically instantaneous stimulation of H^+ excretion in carrot cells upon addition of auxin (Barr *et al.*, 1990b).

If two different mechanisms or sources of proton excretion exist in the plasma membrane of plants, the H^+-ATPase and redox-associated protons, how are they affected by hormones? Data on this point are minimal. When

Table X. The Effect of Plant Growth Regulators on Transmembrane Ferricyanide Reduction and Associated H^+ Excretion

Plant material	Growth regulator	Concentration (μm)	Stimulation or inhibition of H^+ excretion with HCF III (Δ) (%)	Stimulation or inhibition of HCF III reduction (%)	Reference
Maize roots	IAA	10	−100	−100	Böttger and Hilgendorf, 1988
Maize roots (at 21 kPa)	IAA	10	—	+16	
Maize roots (at 1 kPa)	IAA	10	— rate with HBI IV	+110 HBI IV reduction	
Maize roots	2,4-D	10	−38	−100	Lüthje and Böttger, 1989
Maize roots	α-NAA	2	−47	−65	

maize roots were used to measure HCF III, HBI IV, or HCI IV reduction and associated H^+ excretion with a computer-controlled pH redoxstat (Böttger and Hilgendorf, 1988; Lüthje and Böttger, 1989), 100% inhibition by 10 μM IAA of both activities (Table X) was observed, while with 2,4-D and the HBI IV as the electron acceptor its reduction was completely inhibited, while H^+ excretion was inhibited 38%. Some differences in the degree of inhibition were also noted with 2 μM α-NAA (Lüthje and Böttger, 1989). In apical segments of *Cuscuta*, inhibitions of H^+ excretion by growth regulators ranged from 19 to 98% (Revis and Misra, 1988), but comparable data for inhibition of transmembrane ferricyanide reduction are not available. In all the above studies, the concentrations of growth regulators were high (0.1–10 μM), but falling within the range of physiological. With carrot cells, it has been our experience to see stimulation with low concentrations of growth regulators (1–10 nM), but various degrees of inhibition of transmembrane ferricyanide reduction and associated H^+ excretion by concentrations in the range of 1–10 μM. A similar effect of auxin concentrations has also been described on the H^+-ATPase of tobacco leaves; stimulation of the enzyme by low concentrations of auxin, inhibition by higher ones (Santoni *et al.*, 1990). In all cases with cultured carrot or soybean cells, as with an isolated hormone-sensitive NADH oxidase, the hormone effect has been greater on NADH oxidation than on transmembrane ferricyanide reduction [Brightman *et al.*, 1988 and Barr (unpublished data)]. It is not known for sure but implied that the isolated NADH oxidase may also be a transmembrane enzyme, but three subunits of 36, 52, and 72 kDa on SDS-PAGE. Since NADH oxidation can occur on isolated plasma membranes with either

right-side-out orientation (Barr *et al.*, 1985a; Sandelius *et al.*, 1987) or with cytoplasmic side-out membrane vesicles (Barr *et al.*, 1986; Askerlund *et al.*, 1988) or in sealed vesicles with internal NADH (Böttger, 1989), the inner NADH oxidation site can act as an electron donor for transmembrane ferricyanide reduction, which means that the protons liberated in this reaction may be localized in a special membrane domain, as in chloroplasts (Dilley *et al.*, 1987). From here they have three options: (1) they may activate the H^+-ATPase and be excreted through regular H^+ channels; (2) they may be excreted through their own channel, perhaps, with a delay of 5 min, as reported by Rubinstein and Stern (1986) for maize roots; (3) they may acidify the cytoplasm or a special membrane domain and thus act as a signal transmitter to induce growth. It is not possible to distinguish between these three options at present, but since growth regulators stimulate H^+ excretion, option (3) cannot be excluded as a mechanism for induction of growth by growth hormones. If acidification of a special membrane domain occurs after contact with a plant hormone, a signal leading to growth may be transmitted via the phosphoinositol signal cascade.

The Function of H^+ Excretion

If only electrons traverse the plasma membrane to the outside of the cell (Rubinstein and Stern, 1986; Lass *et al.*, 1986; Marré *et al.*, 1988a, b; Ullrich and Guern, 1989, 1990), then the redox-associated protons cannot be engaged in energization of the plasma membrane and in ion and solute transport into the cell. These functions for H^+ are claimed for the plant plasma membrane H^+-ATPase (Serrano, 1985, 1988; Sze, 1985; Pedersen and Carafoli, 1987) as the only mechanism for proton excretion by plant cells. On the other hand, there are studies where the stoichiometry between electrons and H^+ appears to imply a tight coupling in a ratio close to 1 (Federico and Giartosio, 1983; Marigo and Belkoura, 1985; Böttger *et al.*, 1985; Neufeld and Bown, 1987; Bown and Crawford, 1988; Elzenga and Prins, 1989). In *Elodea*, glucose caused a hyperpolarization of membrane potential and stimulation of ferricyanide reduction (Ivankina and Novak, 1988; Novak *et al.*, 1988). It can also be argued that those investigators who find the ratio of H^+ excreted by aquatic plants in relation to ferricyanide reduction short of 1 : 1 but make up the difference with K^+ released (Lass *et al.*, 1986; Marré *et al.*, 1988a, b; Trockner and Marré, 1988; Ullrich and Guern, 1989, 1990) are supporters of ion transport by the plasma membrane, regardless of the proton source. What they see cannot be mediated totally by the plasma membrane H^+-ATPase (Serrano, 1988). In very few examples have the H^+-ATPases been totally inactivated before adding ferricyanide to

see if protons could still be excreted, as shown by Bown and Crawford (1988) with *Asparagus* mesophyll cells.

If the protons excreted by plant cells arise by two different mechanisms, the action of the plasma membrane H^+-ATPase and from redox-associated reactions (Barr, 1988), then there may be two different functions for these protons. The most obvious function for the H^+-ATPase-mediated protons would be their role in cell wall loosening during elongation growth by plants, as predicted by the acid-growth hypothesis (Rayle and Cleland, 1977; Cleland, 1981, 1987; Kutschera and Schopfer, 1985a). However, data by Morré *et al.* (1988) have shown that inhibitors of NADH oxidase, which inhibit plasma membrane redox reactions of isolated plasma membrane vesicles, inhibited elongation growth by soybean hypocotyls to an equal degree. This again, points to different functions for the ATPase-mediated and redox-associated protons.

Since data of studies dealing with H^+ excretion by plant cells are often contradictory, depending on species, age, and assay conditions, no firm conclusions can be drawn in regard to the precise mechanism of H^+ excretion. All sides of the question are presented without bias in hopes that in the future more details on the process of H^+ excretion will be clarified.

References

Alcantara, E., and de la Guardia, M. D. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, p. 410.

Alliotte, T., Tiré, C., Engler, G., Peleman, J., Caplan, A., Van Montagu, M., and Inzé, D. (1989). *Plant Physiol.* **89**, 743–752.

Askerlund, P., Larsson, C., and Widell, S. (1988). *FEBS Lett.* **239**, 23–28.

Barr, R. (1987). In *Redox Functions of the Eukaryotic Plasma Membrane* (Ramirez, J. M., ed.), Consejo Superior de Investigaciones Cientificas, Madrid, pp. 29–40.

Barr, R. (1988). *Physiol. Plant.* **73**, 194–199.

Barr, R., and Crane, F. L. (1990). In *Oxidoreductases at the Plasma Membrane: Relation to Growth and Transport*, Vol. II (Crane, F. L., Morré, D. J., and Löw, H., eds.), CRC Press, Boca Raton, Florida, in press.

Barr, R., Sandelius, A. S., Crane, F. L., and Morré, D. J. (1985a). *Biochem. Biophys. Res. Commun.* **131**, 943–948.

Barr, R., Stone, B., Craig, T. A., and Crane, F. L. (1985b). *Biochem. Biophys. Res. Commun.* **126**, 262–268.

Barr, R., Sandelius, A. S., Crane, F. L., and Morré, D. J. (1986). *Biochim. Biophys. Acta* **852**, 254–261.

Barr, R., Martin, O., Jr., and Crane, F. L. (1987). *Proc. Indiana Acad. Sci.* **96**, 139–144.

Barr, R., Böttger, M., and Crane, F. L. (1990a). *Plant Sci.* **69**, 33–38.

Barr, R., Böttger, M., and Crane, F. L. (1990b). *Proc. Indiana Acad. Sci.*, in press.

Beechey, R. B., Holloway, C. T., Knight, I. G., and Robertson, A. M. (1966). *Biochem. Biophys. Res. Commun.* **23**, 75–80.

Belkoura, M., and Marigo, G. (1986). *Biochemie* **68**, 1299–1302.

Belkoura, M., Ranjeva, R., and Marigo, G. (1986). *Plant Cell Environ.* **9**, 653–656.

Bernstein, M., Dahse, I., Müller, E., and Petzold, U. (1989). *Biochem. Physiol. Pflanzen* **185**, 343–356.

Blein, J.-P., Canivenc, M.-C., De Cherade, X., Bergon, M., Calmon, J.-P., and Scalla, R. (1986). *Plant Sci.* **46**, 77–85.
Böttger, M. (1989). In *Plant Membrane Transport* (Dainty, J., De Michelis, I. M., Marré, E., and Rasi-Caldogno, eds.), Elsevier, Amsterdam, pp. 55–60.
Böttger, M., and Hilgendorf, F. (1988). *Plant Physiol.* **86**, 1038–1043.
Böttger, M., and Lüthen, H. (1986). *J. Exp. Bot.* **37**, 666–675.
Böttger, M., Bigdon, M., and Soll, H. J. (1984). *Z. Pflanzenphysiol.* **114**, 467–475.
Böttger, M., Bigdon, M., and Soll, H. J. (1985). *Planta* **163**, 376–380.
Bown, A. W., and Crawford, L. A. (1988). *Physiol. Plant.* **73**, 170–174.
Bown, A., Crawford, L., and Rodriguez, D. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, pp. 233–242.
Brightman, A. O., Barr, R., Crane, F. L., and Morré, D. J. (1988). *Plant Physiol.* **86**, 1264–1269.
Chalmers, J. D. C., Coleman, J. O. D., and Walton, N. J. (1984). *Plant Cell Rep.* **3**, 243–246.
Cleland, R. E. (1976). *Plant Physiol.* **58**, 210–213.
Cleland, R. E. (1987). In *Plant Hormones and Their Role in Plant Growth and Development* (Davies, P. J., ed.), Martinus Nijhoff Publishers, Dordrecht, pp. 132–148.
Cleland, R. E., and Rayle, D. L. (1978). *Bot. Mag. Special Issue* **1**, 125–139.
Collinge, M., and Trewavas, A. J. (1989). *J. Biol. Chem.* **264**, 8865–8872.
Craig, T. A., and Crane, F. L. (1981). *Proc. Indiana Acad. Sci.* **90**, 150–155.
Craig, T. A., and Crane, F. L. (1982). *Proc. Indiana Acad. Sci.* **91**, 150–154.
Craig, T. A., and Crane, F. L. (1985). In *Current Topics in Plant Biochemistry and Physiology*, Vol. 4 (Randall, D. D., Blevins, B. G., and Larson, R. L., eds.), University of Missouri, Columbia, p. 247.
Crane, F. L. (1989). In *Second Messengers in Plant Growth and Development* (Boss, W., and Morré, D. J., eds.), Alan R. Liss, New York, pp. 115–143.
Crane, F. L., and Barr, R. (1989). In *CRC Critical Reviews in Plant Sciences*, Vol. 8, CRC Press, Boca Raton, Florida, pp. 273–307.
Crane, F. L., Barr, R., Craig, T. A., and Misra, P. C. (1984). In *Proceedings Eleventh Annual Meeting Plant Growth Regulator Society of America* (Cooke, A. R., ed.), Plant Growth Regulator Society, Coral Gables, Florida, pp. 87–95.
Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C., and Löw, H. (1985). *Biochim. Biophys. Acta* **811**, 233–264.
Dahse, I., Bernstein, M., Müller, E., and Petzold, U. (1989). *Biochem. Physiol. Pflanzen* **185**, 145–180.
De Robertis, E. D. P., and De Robertis, E. M. F., Jr. (1987). In *Cell and Molecular Biology*, 8th edn., Lea and Febiger, Philadelphia, pp. 293–323.
De Vos, C. R., Lubberding, H. J., and Bienfait, H. F. (1986). *Plant Physiol.* **81**, 842–846.
del Valle-Tascon, S., Salguero, J., and Gonzalez, F. (1987). *Plant Physiol.* **83**, 148, abstract No. 892.
Dilley, R. A., Theg, S. M., and Beard, W. A. (1987). *Annu. Rev. Plant Physiol.* **38**, 347–389.
Douce, R., and Day, D. A., eds. (1985) In *Encyclopedia of Plant Physiology*, New Series, Vol. 18, Springer-Verlag, Berlin.
DuPont, F. M. (1989). *Plant Physiol.* **89**, 1401–1412.
Elzenga, J. T. M., and Prins, H. B. A. (1989). *Plant Physiol.* **91**, 68–72.
Federico, R., and Giartosio, C. E. (1983). *Plant Physiol.* **73**, 182–184.
Felle, H. (1988). *Planta* **174**, 495–499.
Fischer, E., Weber, A., von Branitz, H. S., and Lüttge, U. (1988a). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, p. 95–104.
Fischer, E., Lüttge, U., and Varanini, Z. (1988b). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, p. 400.
Galtier, N., Belver, A., Gibrat, R., Grouzis, J.-P., Rigaud, J., and Grignon, C. (1988). *Plant Physiol.* **87**, 491–497.
Giannini, J. L., and Briskin, D. P. (1988). *Arch. Biochem. Biophys.* **260**, 653–660.

Grzesiek, S., and Dencher, N. A. (1988). *Biochim. Biophys. Acta* **938**, 411–424.
Guern, J., and Ullrich-Eberius, C. I. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, pp. 253–262.
Guern, J., Mathiew, Y., Ephritikhine, G., Ullrich-Eberius, C. I., Lüttge, U., Marré, M. T., and Marré, E. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, pp. 413–414.
Guilfoyle, T. J. (1986). *CRC Crit. Rev. Plant Sci.* **4**, 247–276.
Hagen, G. (1987). In *Plant Hormones and Their Role in Plant Growth and Development* (Davis, P. J., ed.), Martinus Nijhoff Publishers, Dordrecht, pp. 149–163.
Hagen, G., Kleinschmidt, A., and Guilfoyle, T. J. (1984). *Planta* **162**, 147–153.
Hager, A., Menzel, H., and Krauss, A. (1971). *Planta* **100**, 47–75.
Hassidim, M., Rubinstein, B., Lerner, H. R., and Reinhold, L. (1987). *Plant Physiol.* **85**, 872–875.
Henderson, R. M., and Graf, J. (1988). In *pH Homeostasis. Mechanisms and Control* (Häusinger, D., ed.), Academic Press, London, pp. 5–26.
Heytler, P. G. (1979). In *Methods in Enzymology*, LV, *Biomembranes*, Part F: *Bioenergetics —Oxidative Phosphorylation* (Fleischer, S., and Packer, L., eds.), Academic Press, New York, pp. 462–472.
Ivankina, N. G., and Novak, V. A. (1980). In *Plant Membrane Transport: Current Conceptual Issues* (Spanswick, R. M., Lucas, W. J., and Dainty, J., eds.), Elsevier, Amsterdam, pp. 503–504.
Ivankina, N. G., and Novak, V. A. (1988). *Physiol. Plant.* **73**, 161–164.
Ivankina, N. G., Novak, V. A., and Miclashevich, A. I. (1984). In *Membrane Transport in Plants* (Cram, W. J., Janácek, K., Rybová, R., and Sigler, K., eds.), Wiley-Interscience, New York, pp. 404–405.
Jagendorf, A. T. (1977). In *Encyclopedia of Plant Physiology*, New Series, Vol. 5 (Trebst, A., and Avron, M., eds.), Springer-Verlag, Berlin, pp. 307–337.
Kochian, L., and Lucas, W. J. (1985). *Plant Physiol.* **77**, 429–436.
Komor, E., Thom, E., and Maretzki, A. (1987). *Planta* **170**, 34–43.
Kutschera, U., and Schopfer, P. (1985a). *Planta* **163**, 483–493.
Kutschera, U., and Schopfer, P. (1985b). *Planta* **163**, 494–499.
Lass, B., Thiel, G., and Ullrich-Eberius, C. I. (1986). *Planta* **169**, 251–259.
Lee, H. C., Forte, J. G., and Epel, D. (1982). In *Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions* (Nucitelli, R., and Deamer, D. W., eds.), Alan R. Liss, New York, pp. 135–160.
Löppert, H. (1983). *Planta* **159**, 329–335.
Lüthen, H., and Böttger, M. (1988). *Plant Sci.* **54**, 37–43.
Lüthje, S., and Böttger, M. (1989). *Biochim. Biophys. Acta* **977**, 335–340.
Lüttge, U., and Clarkson, D. T. (1985). *Prog. Bot.* **47**, 73–86.
Marigo, G., and Belkoura, M. (1985). *Plant Cell Rep.* **4**, 311–314.
Marré, E., and Ballarin-Denti, A. (1985). *J. Bioenerg. Biomembr.* **17**, 1–21.
Marré, E., Lado, P., Rasi-Caldogno, F., and Colombo, R. (1973). *Plant Sci.* **1**, 179–184.
Marré, E., Marré, M. T., Albergoni, F. G., Trockner, V., and Moroni, A. (1988a). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, pp. 233–241.
Marré, M. T., Moroni, A., Albergoni, F. G., and Marré, E. (1988b). *Plant Physiol.* **87**, 25–29.
McCarty, R. E. (1977). In *Encyclopedia of Plant Physiol.*, New Series. Vol. 5 (Trebst, A., and Avron, M., eds.), Springer-Verlag, Berlin, 437–447.
Møller, I. M., and Lin, W. (1986). *Annu. Rev. Plant Physiol.* **37**, 309–334.
Morré, D. J., Navas, P., Penel, C., and Castillo, F. J. (1986). *Protoplasma* **133**, 195–197.
Morré, D. J., Brightman, A. O., Wu, L.-Y., Barr, R., Leak, B., and Crane, F. L. (1988). *Physiol. Plant* **73**, 187–193.
Neufeld, E., and Bown, A. W. (1987). *Plant Physiol.* **83**, 895–899.
Novak, V. A., and Ivankina, N. G. (1983). *Sov. Plant Physiol.* **30**, 845–853 (in English).

Novak, V. A., Ivankina, N. G., Morokova, E. A., and Miclashevich, A. I. (1988). *Physiol. Plant.* **73**, 165–169.
Parthier, B. (1989). *Biochem. Physiol. Pflanzen* **185**, 389–414.
Pedersen, P. L., and Carafoli, E. (1987). *Trends Biochem. Sci.* **12**, 146–150.
Pope, A. J., and Leigh, R. A. (1988). *Plant Physiol.* **86**, 1315–1322.
Pugin, A. Y., Tirilly, Y., and Barbier-Brygoo, H. (1988). *Plant Physiol. Biochem.* **26**, 347–356.
Ray, P. M. (1987). In *Physiology of Cell Expansion* (Cosgrove, D. J., and Knievel, D. P., eds.), Amer. Soc. Plant Physiol., Rockville, Maryland, pp. 1–17.
Revis, S., and Misra, P. C. (1988). *Biochem. Physiol. Pflanzen* **183**, 487–494.
Römheld, V., Müller, C., and Marschner, H. (1984). *Plant Physiol.* **76**, 603–606.
Rubinstein, B., and Stern, A. I. (1986). *Plant Physiol.* **80**, 805–811.
Sandelius, A. S., Barr, R., Crane, F. L., and Morré, D. J. (1987). *Plant Sci.* **48**, 1–10.
Santoni, V., Vansuyt, G., and Rossignol, M. (1990). *Plant Sci.* **68**, 33–38.
Senn, A. P., and Goldsmith, M. H. M. (1988). *Plant Physiol.* **88**, 131–138.
Serrano, R. (1985). In *Plasma Membrane ATPase of Plants and Fungi*, CRC Press, Boca Raton, Florida, pp. 80–129.
Serrano, R. (1988). *Biochim. Biophys. Acta* **947**, 1–28.
Sijmons, P. C., and Bienfait, H. F. (1986). *Biochem. Physiol. Pflanzen* **181**, 283–299.
Sijmons, P. C., van den Briel, W., and Bienfait, H. F. (1984). *Plant Physiol.* **75**, 219–221.
Sze, H. (1985). *Annu. Rev. Plant Physiol.* **36**, 175–208.
Theologis, A. (1986). *Annu. Rev. Plant Physiol.* **37**, 407–438.
Theologis, A., Huynh, T. V., and Davis, R. W. (1985). *J. Mol. Biol.* **183**, 53–68.
Thiel, G., and Kirst, G. O. (1988). *J. Exp. Bot.* **39**, 641–654.
Thom, M., and Maretzki, A. (1985). *Plant Physiol.* **77**, 873–876.
Trockner, V., and Marré, E. (1988). *Plant Physiol.* **87**, 30–35.
Ullrich, C. I., and Guern, J. (1989). In *Plant Membrane Transport, the Current Position* (Dainty, J., De Michelis, I. M., Marré, E., and Rasi-Cadogno, F., eds.), Elsevier, Amsterdam, pp. 387–388.
Ullrich, C. I., and Guern, J. (1990). *Planta* **180**, 390–399.
van der Zaal, E. J., Memelink, J., Mennes, A. M., Quint, A., and Libbenga, K. R. (1987). *Plant Mol. Biol.* **10**, 145–157.
Walker, J. C., Legocka, J., Edelman, L., and Key, J. L. (1985). *Plant Physiol.* **77**, 847–850.
Xia, J.-H., and Saglio, P. (1990). *Plant Physiol.* **93**, 453–459.